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Differences in winter cold hardiness reflect the geographic range disjunction of *Neophasia menapia* and *Neophasia terlooii* (Lepidoptera: Pieridae)

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ABSTRACT

Predicting how rapid climate change will affect terrestrial biota depends on a thorough understanding of an organism's biology and evolutionary history. Organisms at their range boundaries are particularly sensitive to climate change. As predominantly terrestrial poikilotherms, insects are often geographically limited by extremes in ambient temperatures. We compared the cold hardiness strategies of two geographically widespread butterflies, the pine white, *Neophasia menapia*, and the Mexican pine white, *N. terlooii* (Lepidoptera: Pieridae), at the near-contact zone of their range boundaries. Eggs are laid on pine needles and are exposed to harsh winter conditions. Eggs were collected from wild-caught butterflies, and we determined the supercooling point (SCP) and lower lethal temperature (LLT_{50}) of overwintering eggs. The SCP of *Neophasia menapia* eggs (−29.0 ± 0.6 °C) was significantly lower than that of *N. terlooii* eggs (−21.8 ± 0.7 °C). Both species were freeze-intolerant and capable of surviving down to their respective SCPs (LLT_{50} of *N. menapia* between −30 and −31 °C, *N. terlooii* between −20 and −21 °C). Cold exposure time did not affect the survival of *N. menapia*, but *N. terlooii* experienced somewhat greater mortality at sub-freezing temperatures during longer exposures. Our results, coupled with an analysis of microclimate data, indicate that colder winters in northern Arizona may contribute to the northern range limit for *N. terlooii*. Furthermore, careful analysis of historical weather data indicates that mortality from freezing is unlikely in southern Arizona but possible in northern Arizona. Movements of *Neophasia* range boundaries could be monitored as potential biological responses to climate change.

1. Introduction

Ambient environmental temperatures greatly affect the distribution of organisms. As small-bodied poikilotherms, insects are particularly affected by temperature (Sinclair et al., 2003), and low temperatures have a strong influence on insect distributions (Shi et al., 2012). Range boundaries have been shown to move north and upward in elevation in response to warming climate (Parmesan et al., 1999; Konvicka et al., 2003; Scriber, 2011). As such, movements of insect range boundaries can be useful indicators of biological responses to climate change.

The western North American arid regions are a natural laboratory for studying the influence of historical climate changes on the diversification of terrestrial biota (Smith and Farrell, 2005). Range expansions and contractions are a fairly common occurrence in the sky island region (Felger and Wilson, 1994), which encompasses the mountains of north-central Mexico and southeastern Arizona. Within this region, we focused on two butterflies: the pine white butterfly, *Neophasia menapia* (C. Felder and R. Felder, 1859), and the Mexican pine white butterfly, *N. terlooii* Behr, 1869. *Neophasia menapia* ranges from southwestern British Columbia to Guadalupe Mountains National Park in southwest Texas (Lotts and Naberhaus 2016; Scott 1986). *Neophasia terlooii* ranges from the sky islands of southeastern Arizona to the Sierra Madre of central Mexico (Bailowitz and Brock, 1990) (Fig. 1). The ranges of *N. menapia* and *N. terlooii* meet in central Arizona, with the former in the White Mountains and the latter in the sky islands, but there is no geographic overlap.

Mountain habitats for *Neophasia* spp. are susceptible to subfreezing conditions, with temperatures as low as −35 °C (National Centers for Environmental Information, 2016). In Arizona, *N. menapia* is univoltine and lays its eggs in July and August, while *N. terlooii* is bivoltine with the second generation laying its eggs in October. Eggs of both species are oviposited in linear clusters and overwinter on live pine needles.

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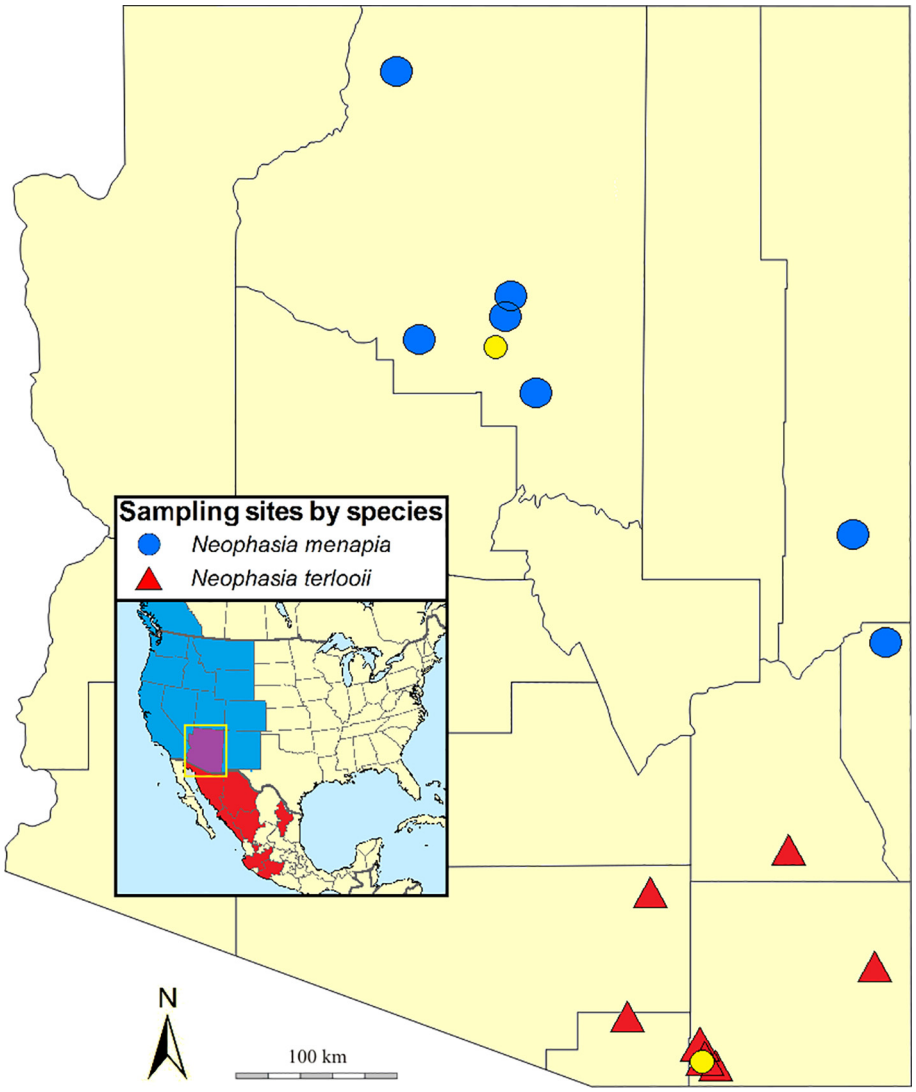

Fig. 1. Sampling sites for *Neophasia* in Arizona, with inset map showing geographic range of both species by state/province (there was one occurrence of *N. menapia* in extreme SW Texas): *Neophasia menapia* in blue and *N. terlooii* in red. Arizona is in purple to indicate the near-contact zone of both species. On the foreground Arizona map, blue circles indicate sampling sites for *Neophasia menapia*. Red triangles indicate sampling sites for *N. terlooii*. Yellow circles indicate where iButton temperature data were collected, with the northernmost circle representing two trees in Flagstaff and the southernmost circle representing one tree in Sawmill Canyon. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Pine forests are known to be sensitive to climate change (Allen and Breshears, 1998), but the long generation time of trees makes them poor indicators of rapid, ongoing responses to recent climate change. Thus, *Neophasia* spp., with their close association with pine trees, coupled with their abundance, ease of detection, and rapid annual life cycles, are prime candidates for indicators of biological responses to rapid climate change among insects in pine forests.

We investigated the cold hardiness strategy and limits of survival for both *Neophasia* spp. Among insects, three cold hardiness strategies are typically recognized (Lee, 2010): 1) Freeze-tolerant species can survive internal ice formation, 2) Freeze-intolerant species must remain in a supercooled state and are killed by any internal ice, and 3) Chill-susceptible species succumb to cold injury well above the supercooling point (SCP; i.e., the temperature at which internal ice nucleation occurs). A majority of insects are incapable of freezing and depress their supercooling point by removing potential ice nucleating agents, synthesizing cryoprotectants and, in some cases, producing antifreeze proteins (Lee 2010). By comparing the SCP to the lower lethal temperature (e.g., LLT_{50}, the temperature at which 50% of individuals die), the cold hardiness strategy can be empirically determined (Sinclair et al., 2015). Intensity and duration of exposure interact to determine survival – prolonged exposure to temperatures above the SCP can result in mortality, even in freeze-intolerant insects with low SCPs (Delisle et al., 2013).

Cold hardiness can limit the geographic ranges of insects (Shi et al., 2012). In our system, two congeners have disjunct distributions near a major ecotone, despite the fact that the host plants for both species overlap at this ecotone (Southwest Environmental Information Network, 2016). Our goal was to investigate differences in cold hardiness and compare them to differences in temperature profiles at the ecotone. We propose that a lower level of cold hardiness in *N. terlooii* may contribute to its northern range limit. We then use the results of this study to discuss mechanisms of range disjunction and implications for using *Neophasia* as bioindicators of the effects of climate change on forest-dwelling insects. *Neophasia* spp. are ubiquitous members of pine forest communities in western North America, at times reaching outbreak numbers (Ciesla, 1974), and our study is the first to empirically investigate their thermal biology.

2. Materials and methods

2.1. Collecting adult specimens

Sampling took place at similar elevations and latitudes in Arizona, where the ranges of both species are in closest proximity. To account for cold hardiness variations within each species, butterflies were collected from seven sites along their range boundaries (Fig. 1). Butterflies used for egg production were collected during the peak flight seasons of adults in 2013–2015: July/August for *N. menapia* and October for *N. terlooii*. Field-collected butterflies were stored individually in glassine

envelopes and transported to the laboratory, where groups of up to five females were placed into containers lined with a damp paper towel. The containers were placed into a refrigerator for 24 h in complete darkness to acclimate butterflies to captivity while minimizing stress and exertion associated with escape behavior. Refrigerator temperatures were adjusted to approximate mean night time lows during the season of collection for each species: 12.8 °C for *Neophasia menapia* and 5 °C for *N. terlooii*.

2.2. Obtaining eggs

Following the 24-h acclimation period, *N. menapia* females were returned to ambient conditions and individually placed into 30.5 × 30.5 × 30.5-cm screen cages (The Caterpillar Castle, livemonarch.com). A single cutting of *Pinus ponderosa* ssp. *scopulorum* (Engelmann) was placed in each cage for oviposition. The room had windows which allowed for a natural photoperiod and room temperatures ranged from 13 °C (night) to 28 °C (day). Pine needles were checked for eggs every evening. If eggs were present, the entire needle fascicle was removed and placed into a 50-dram vial. Butterflies were misted daily and fed fruit punch Gatorade® (a sugary sports drink supplemented with electrolytes) every other day to increase longevity. To collect eggs from *N. terlooii*, females were individually placed into 710-mL 11 × 16 × 7-cm plastic food storage containers following their 24-h acclimation period. Five to eight needle fascicles of *Pinus engelmanii* Carrière, *P. ponderosa* ssp. *arizonica* (Engelmann), or a combination of the two were placed into each container, and containers were kept at a natural photoperiod. Room temperatures varied from 20 to 26.5 °C. Butterflies were fed every other day. Needle fascicles with eggs were removed each evening and placed into vials.

2.3. Shipment of eggs

At the end of each field season, egg vials were packaged into a cooler and were transported to the University of Florida. Temperatures during shipment ranged from 18 to 27.5 °C for *N. menapia* in August and from 1 to 10 °C for dormant (see next section) *N. terlooii* in October. Eggs of *N. terlooii* in dormancy were immediately transferred to a refrigerator held at 5 °C. Eggs of *N. menapia* that had not yet been placed into refrigeration were kept at room temperature (21.5 to 23 °C) and kept on a 13L:11D late summer photoperiod.

2.4. Dormancy initiation for cold hardiness assays

Overwintering conditions typically don't materialize for *N. menapia* until mid-September, so their eggs were held at room temperature for either 30 (LLT_{50} assay) or 45 d (SCP assay) before being acclimated to 5 °C for 1–2 mo prior to the assays. If eggs were never exposed to simulated winter conditions, they never hatched and eventually died, suggesting that they were in diapause (Kostál, 2006). Eggs were lightly misted with water weekly to prevent desiccation. Eggs of *Neophasia terlooii*, which were collected later in the season, were held at room temperature for 24 h then transferred to a 5 °C refrigerator for 1–2 mo prior to SCP and LLT_{50} assays. This quick transfer to cold conditions was done because, in a preliminary assay, a small cohort of eggs left at room temperature and natural photoperiod began to hatch after several days. Another small cohort of eggs under a photoperiod and temperature regime designed to mimic natural conditions did not hatch until early February. These observations suggest that *N. terlooii* eggs were in quiescence maintained by cool late fall and winter nights. There are no collection records of adult *N. terlooii* until June, therefore the October generation is likely the one that overwinters. Henceforth, dormancy will refer to either the diapause of *N. menapia* or the quiescence of *N. terlooii*.

2.5. Supercooling point assay

The SCPs of *N. menapia* were measured in eggs collected in the summer of 2013 while the SCPs of *N. terlooii* were measured in eggs collected in October 2013. Measurements were made throughout the 4-mo dormancy for each species, from October 2013 through January 2014 for *N. menapia* and from November 2013 through February 2014 for *N. terlooii*. Due to the linear pattern of oviposition on a pine needle (ranging from two to 21 eggs in a line), neighboring eggs could impact the thermal environment via conduction. Therefore, we kept eggs in small groups for thermal treatments to retain conductive influences, but limited the size of the groups to three eggs to maximize replicates. Egg clusters were selected at random for each treatment, with two to four clusters in each treatment.

To measure SCP, vacuum grease was used to secure a type T thermocouple to the middle egg in each cluster. A refrigerated 95% ethanol bath (Neslab RTE 740, Thermo Fisher Scientific, Newington, NH) was initially set to 5 °C and decreased at 0.1 °C/min until the freezing exotherm was observed. The temperature the egg registered immediately before the exotherm spike was recorded as the SCP (Fig. S1). Our cooling rate was based on microclimate data, where iButton dataloggers in trees reported some nights of cooling at roughly equivalent rates. The egg connected to the thermocouple always registered the highest spike out of the three eggs in the cluster, and the SCP of this egg was used in analyses.

After all three eggs in each cluster registered an SCP, the vial was removed from the bath and placed at room temperature for 5 min, at which point the eggs had warmed to 5 °C, and were transferred back to the refrigerator. Eggs experienced temperatures of up to 5 °C below their SCPs before they were removed. After 4 mo of dormancy conditions, eggs were removed from refrigeration, placed on the lab counter, and exposed to room temperatures (21.5 to 23 °C) and a 13L:11D spring photoperiod. Eggs were observed for hatching for 1 mo. Control eggs remained in dormancy for 4 mo, then were allowed to hatch. Dead eggs were dissected to check for any signs of development into pharate first instar larvae prior to death.

2.6. Lower lethal temperature assay

All LLT_{50} measurements were taken during the last 2 mo of dormancy from eggs collected in 2014 and 2015. This timing was selected because the peak in cold hardiness, as suggested by the lowest SCP measurements, occurred roughly 2 mo into the 4-mo dormancy. A matrix of temperature and time treatment combinations was generated for each species. Each temperature, in increments of 1 °C, was paired with a randomly selected amount of time within a range specific to each temperature. Exposure to lower temperatures occurred for shorter durations to approximate a typical exposure time to that temperature in the wild. Preliminary assays indicated that neither species can survive temperatures lower than its SCP, and therefore we set the lower range limit to 2–5 °C colder than the lowest SCP for each species to ensure that the most cold hardy individuals in each sample would freeze. For cold exposure, eggs were glued to a wooden dowel and housed in a glass test tube, and a type-T thermocouple was placed inside each test tube to monitor cold exposure. For *N. menapia*, temperatures ranged from −10 to −35 °C and times ranged from 5 to 770 min depending on temperature. Egg clusters were randomly assigned to treatments until all treatments in the matrix had been administered and each cluster was only treated once. The same procedure was applied to *N. terlooii* egg clusters, except temperatures ranged from −10 to −30 °C and times ranged from 10 to 870 min. Cooling and warming rates were the same as those in the SCP assay. After cold exposure, eggs were returned to dormancy conditions in the same manner as the SCP assay and then monitored for hatching after the winter. Groups of control eggs remained in dormancy for 4 mo, then were allowed to hatch.

2.7. Tree microclimates

We measured microclimate temperatures at hourly intervals on each of three pine trees, two *P. ponderosa* ssp. *scopulorum* in Flagstaff, AZ, and one *P. englemanii* in Sawmill Canyon, AZ (Fig. 1). An iButton sensor (DS1922L#F50, Maxim Integrated, San Jose, CA) was suspended to an outer tree branch, with four sensors placed in the crown and four in the lowest branches of each tree. At each level, there was one sensor at each of the four cardinal directions. Sensors were put up in July 2014 and taken down July 2015.

2.8. Historical temperature data

Historical temperature data from the past 103 yr were gathered for sites throughout Arizona using the online National Centers for Environmental Information interactive map. Data included daily highs and lows. Sites were chosen that were at similar elevations (~2,350 m) and the closest (5–20 km) available to our collecting sites. There were four sites in the White mountains of northern Arizona and two sites from the Santa Catalina Mountains of southeastern Arizona. Data were gathered from one additional site at Flagstaff Pulliam Airport from July 2014 to July 2015 for comparison with the tree microclimate data gathered 6.3 km from there.

2.9. Statistics

Data were imported into R 3.3.3 (R Core Team 2017), and linear and linear mixed models were used for analyses. To better meet normality criteria for the ANOVA, SCP data were grouped into three age classes based on the number of days into dormancy: 16–49 d (early), 67–93 d (middle), and 110–130 d (late). The SCP of the middle egg in each cluster was the response variable and age, species, and their interaction were the fixed effects. Although we randomly selected eggs from our sampling sites, we also investigated the intraspecific effect of collection site in separate linear models for each species. An ANOVA was then run on each fit model to obtain p-values. A Welch Two Sample t-was run on SCP data pooled from all test periods to determine the effect of species on SCP.

Because we varied both time and temperature independently, traditional regression-based methods for LLT_{50} estimation could not be applied to our dataset. Instead, we estimated LLT_{50} as the lowest temperature producing at most 50% survival, regardless of exposure time. Each egg in a cluster was scored either 0 for not hatching or 1 for hatching, and mean scores for each cluster (i.e., proportion hatching) were analyzed. Baseline mortality of control groups was accounted for by performing a Schneider-Orelli correction on each egg's survival score. Both species were initially analyzed together to determine any species effects. In the fit linear mixed model, proportion hatching was the response and the exposure temperature, exposure time, species, time and species interaction, and temperature and species interaction were the fixed effects. Mother and egg cluster nested within mother were the random effects. Due to a significant species effect on survival and a species interaction effect with time and temperature, both species were then analyzed separately to more clearly assess the effects of time and temperature on proportion hatching. In the fit linear mixed models for each species, proportion hatching was the response and the exposure temperature, exposure time, and time and temperature interaction were the fixed effects. Mother and egg cluster nested within mother were the random effects. ANOVAs were run on each fit model to obtain p-values.

Microclimate data were analyzed for all trees together and for each tree independently to test the effects of vertical profile (either crown or lower branches) and aspect (either of the four cardinal directions) on temperature. Temperature data were analyzed for hourly differences and for nightly minimum differences. In the linear model analyzing all trees together, temperature was the response and tree, vertical profile, aspect, and region (i.e., tree(s) in *N. menapia* habitat or *N. terlooii* habitat) were the fixed effects. In the linear models for each tree, temperature was the response and vertical profile and aspect were the fixed effects. In all models, the time effects, i.e., hourly or nightly minimum temperatures, were incorporated as POSIXct covariates or date covariates, respectively. Because we were only interested in the coldest times of the year, only data from the months of October through March were analyzed.

The four historical climate data sites in northern Arizona were grouped into a category to represent *N. menapia* habitat and the two sites from southern Arizona were grouped into a category to represent *N. terlooii* habitat. A linear model was used to test for the effect of habitat on temperature. Site was also included as a fixed effect. The time effect, i.e., the interval of nightly minimum temperatures, was incorporated into the models as a date covariate. Because we were only interested in the coldest times of the year, only data from the months of October through March were analyzed. The number of times nightly minimums dropped below the supercooling points of either *Neophasia* spp. were determined by manually sorting the data. Lastly, a linear regression was run in which the mean nightly minimums for the two trees in Flagstaff were plotted as a function of the nightly minimums from Flagstaff Pulliam Airport.

3. Results

3.1. Supercooling points

Neophasia menapia supercooled to a lower temperature than *N. terlooii* ($t = -8.5$, d.f. = 37, $p < 0.001$). The mean SCPs were −29.0 °C (range: −33.3 to −22.4 °C) for *N. menapia* (n = 23) and −21.8 °C (range: −25.3 to −18.3 °C) for *N. terlooii* (n = 18). There were no intraspecific differences in SCP with respect to collecting site. The SCP

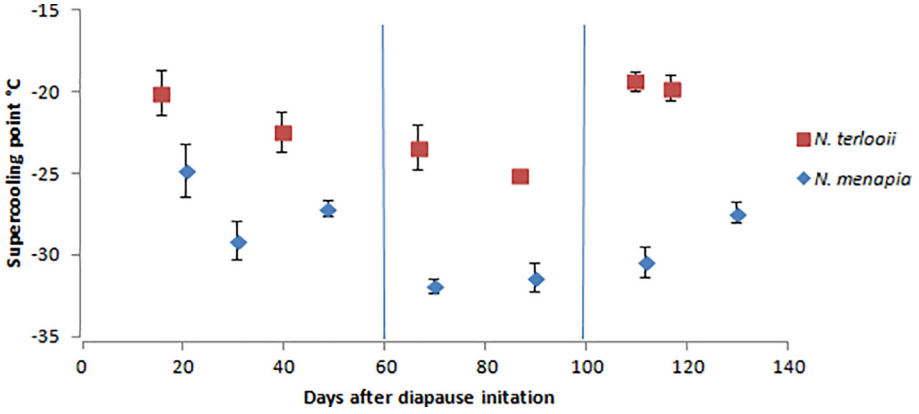

Fig. 2. Clusters of eggs were chilled until they froze. Points on the graph represent the mean supercooling point for two to four clusters of eggs at a particular time in dormancy. Data points are means ± SEM. Vertical blue lines separate the points into age classes for the statistical analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

values for both species changed in the same manner over the course of dormancy, where SCPs were lowest in the middle of dormancy ($F = 12.6$, d.f. = 2;38, $p < 0.001$) (Fig. 2). For either species, none of the eggs that froze after supercooling hatched after being brought out of dormancy (i.e., removed from refrigeration and held at 21.5 to 23 °C under a 13L:11D photoperiod). There were 26 control eggs of *N. menapia* that were held at 5 °C during dormancy, of which 27% hatched and 46% showed signs of embryonic development after being brought out of dormancy but did not hatch. There were 19 control eggs of *N. terlooii* that were held at 5 °C during dormancy, of which 42% hatched and 16% showed signs of embryonic development after being brought out of dormancy but did not hatch. Untreated eggs dissected during dormancy did not develop into pharate first instar larvae until after they were brought out of dormancy.

3.2. Lower lethal temperatures

The LLT_{50} for *N. menapia* was between −30 and −31 °C. All eggs that froze within this temperature range died, while at −30 °C, 57% of the total eggs hatched. Providing eggs did not freeze, neither exposure time, temperature, nor their interaction affected hatching success of *N. menapia* (n = 102) (Fig. 3a). Increased exposure time ($F = 7.9$, d.f. = 1;88, $p = 0.006$) or decreased temperature ($F = 79.8$, d.f. = 1;88, $p < 0.001$) significantly reduced hatching success for *N. terlooii* (n = 93) (Fig. 3b). The LLT_{50} for *N. terlooii* was between −20 and −21 °C. All eggs that froze died, and only eggs exposed to −21 °C or lower froze. Hatching success tended to decrease with greater exposure time at temperatures between −18 and −24 °C, but overall there was not a significant interaction between temperature and exposure time. Of the 135 control eggs of *N. menapia*, 42% hatched and 21% showed signs of development after being brought out of dormancy but did not hatch. Of the 105 control eggs of *N. terlooii*, 71% hatched and 16% showed signs of development after being brought out of dormancy but did not hatch. *Neophasia terlooii* eggs had an overall higher hatching success than *N. menapia* eggs ($F = 39.0$, d.f. = 1;177, $p < 0.001$).

3.3. Tree microclimates

Each tree differed with respect to hourly ($F = 1101.4$, d.f. = 2;78607, $p < 0.001$) and nightly ($F = 531.4$, d.f. = 2;3259, $p < 0.001$) minimum temperatures. Hourly temperatures of tree crowns were consistently warmer than basal branches ($F = 133.7$, d.f. = 1;78607, $p < 0.001$), but not all trees had consistently higher nightly minimums at the crown ($F = 2.4$, d.f. = 1;3259, $p = 0.12$) (Table 1). Although no consistent trends were observed across trees, aspect had a significant effect on hourly temperature ($F = 14.8$, d.f. = 6;78607, $p < 0.001$) but not nightly minimums. On an hourly basis, northern trees were on average 3.6 °C cooler than the southern tree ($F = 44401.2$, d.f. = 1;78608, $p < 0.001$) and, when looking at nightly minimums, northern trees were on average 2.9 °C cooler than the southern tree ($F = 147.9$, d.f. = 1;3259, $p < 0.001$). Tree temperatures never dropped below the supercooling points of either *Neophasia* spp. between October 2014 and March 2015.

Table 1
A comparison of tree microclimates with means from hourly and nightly temperature readings, and a comparison of the coldest temperatures reached. Three trees were each outfitted with eight iButton temperature loggers that recorded temperatures every hour from October 2014 to March 2015. Four iButtons were placed in the crown and four in the basal branches. Asterisks indicate significant within-tree differences between crowns and bases. There is no test for absolute mins.

Habitat	Tree	Tree position	Hourly °C	Nightly min °C	Absolute min °C
Northern	1	Crown	6.0*	0.85*	−12.00
		Base	5.2*	0.21*	−12.50
Northern	2	Crown	3.8*	−3.78	−18.58
		Base	3.4*	−3.82	−19.65
Southern	3	Crown	8.8*	2.32	−10.49
		Base	7.9*	2.08	−10.51

3.4. Historical data

Historical data from the past 103 yr indicated that the northern sites in the range of *N. menapia* were significantly cooler than the southern sites in the range of *N. terlooii* ($F = 3176.4$, d.f. = 1;52050, $p < 0.001$). There were also site variations within each habitat ($F = 517.16$, d.f. = 5;52050, $p < 0.001$). Northern sites had a mean October to March minimum temperature of −6.5 °C and southern sites had a mean October to March minimum temperature of −1.8 °C. Historical all-time minimums differed to a greater degree: −35.6 °C for a northern site in 1979 and −21.7 °C for a southern site in 1963 and 1985, respectively. Over the span of 103 years, air temperatures in the northern habitat dropped down to or below the mean SCP of *N. menapia* (−29.0 °C) 33 times and to or below that of *N. terlooii* (−21.8 °C) 561 times. In the southern habitat, air temperature never dropped below the mean SCP of either species. Tree surface temperatures from October 2014 through March 2015 closely tracked that of air temperatures at Flagstaff airport ($y = 0.8731x + 1.023$, $R^2 = 0.923$, $F = 2152$, d.f. = 1;180, $p < 0.001$) and tended to be slightly warmer than air temperatures (Fig. 4). This result suggests that air temperatures need to

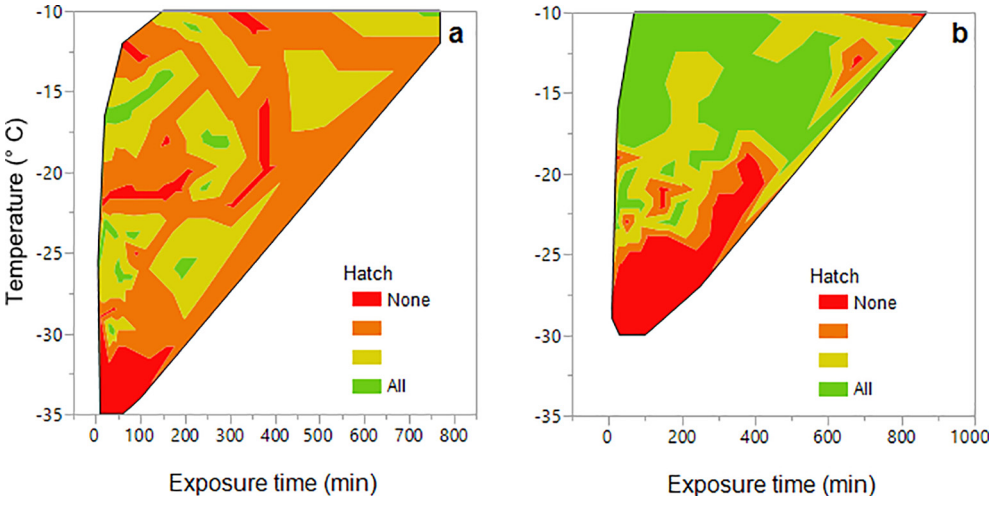

Fig. 3. Clusters of eggs were exposed to various temperatures for different durations. The colored heat maps indicate low hatching success in red and high hatching success in green. The figures were generated in JMP version 12 and colors correspond to contour model values based on the control mortality-corrected data. a) *Neophasia menapia* hatching success. b) *Neophasia terlooii* hatching success. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

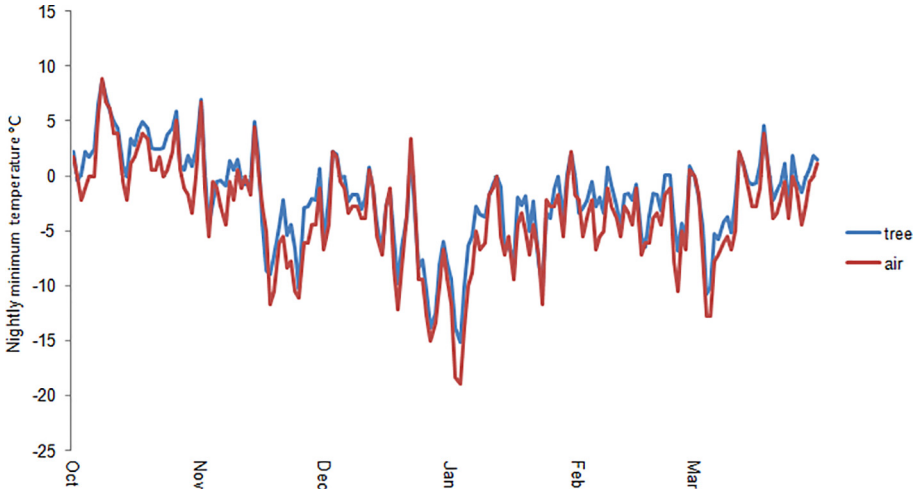

Fig. 4. A comparison of nightly minimum air and tree surface temperatures. The blue line represents the average nightly minimum temperatures from 12 iButton sensors placed on the outer branches of two pine trees in Flagstaff, AZ. The red line represents the nightly minimum air temperatures at the nearby Flagstaff airport. Data span from October 2014 to the end of March 2015. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

be colder than the mean SCPs of *Neophasia* spp. in order for tree surface temperatures to reach each species' SCP. Accordingly, when using the regression equation to calculate the air temperatures necessary for tree surface temperatures to reach the SCPs of either *Neophasia* spp., air temperatures in the northern habitat would have reached those points nine times for *N. menapia* and 323 times for *N. terlooii*.

4. Discussion

Neophasia menapia and *N. terlooii* show distinct, non-overlapping ranges, and we tested whether differences in cold hardiness are consistent with range disjunction in this complex. Both species had lower lethal temperatures that exceeded the nightly minimum temperatures recorded in our study sites, indicating that they are well-adapted to the cold, temperate climates characteristic of Sierra Madre and Rocky Mountains biomes. Tolerance of extreme low temperatures was probably under selection during poleward movement of the common ancestor of *Neophasia* and *Eucheira*, which, according to Braby et al (2007), moved from South America into central Mexico, then *Neophasia* eventually spread into western North America. We found that the northern species, *N. menapia*, was considerably more cold tolerant than southern *N. terlooii*, as indicated by both SCP and LLT_{50} measurements. These observed differences in winter cold hardiness, in conjunction with other mechanisms, may be contributing to allopatry. However, we acknowledge that cold hardiness studies with additional species of insects are needed to rigorously test the hypothesis that differences in cold hardiness lead to allopatry.

Both *Neophasia* spp. had LLT_{50}s that were fairly close to their SCPs, and eggs from neither species were capable of hatching after freezing. Thus, we conclude that both species are freeze-intolerant and that the SCP is a suitable measure of cold hardiness. The LLT_{50} of *N. terlooii* was slightly warmer than the SCP, indicating that *N. terlooii* is mildly chill-susceptible, whereas the LLT_{50} of *N. menapia* was roughly the same as the SCP, indicating *N. menapia* can be classified as freeze-avoidant. Freeze-tolerant insects generally have a higher SCP than freeze-intolerant insects (Turnock and Fields, 2005), and the SCPs of *Neophasia* spp. are consistent with other freeze-intolerant insects. The duration of exposure to sub-freezing temperatures is also important for survival because freeze-intolerant insects can experience freezing or pre-freeze mortality with increased duration at temperatures above the SCP (Delisle et al., 2013). The influence of exposure time on survival was evident in *N. terlooii*, where, at longer exposure times, there was nearly 100% mortality at temperatures several degrees above the mean SCP (−18 to −24 °C and 250 to 450 min) (Fig. 3b). The majority of *N. terlooii* mortality can be attributed to the eggs freezing, as 75% of eggs that died produced a freezing exotherm. In contrast, only 20.1% of *N. menapia* eggs died from freezing at our test temperatures.

In addition to intraspecific variations, responses to low temperatures can vary with ontogeny (Boychuk et al., 2015; Cavieres et al., 2016). We demonstrated a gradual lowering of the SCP after several weeks of dormancy, followed by a gradual rise in SCP toward the end. This result is consistent with seasonal acclimation and has been previously demonstrated in other freeze-intolerant insects (Crosthwaite et al., 2011; Khani and Moharramipour, 2010). These shifts in SCP often result from cryoprotectant production (Crosthwaite et al., 2011; Somme, 1982), changes in water content (Lee, 2010), antifreeze protein production (Bale, 1996, Lee, 2010), and/or removal ice nucleators (Bale, 1996, Lee, 2010). Interestingly, in our assay, the changes in SCP occurred in the absence of environmental cues that would have otherwise indicated a changing season during dormancy, suggesting that responses were endogenously programmed. The reduction in cold tolerance at the end of winter may minimize metabolic costs associated with maintaining high cryoprotectant levels. For example, maintaining high levels of cold hardiness in flies during repeated cold exposures protects against cold injury but significantly reduces their reproductive output (Marshall and Sinclair, 2010).

Both species have cold hardiness that is well matched to low temperature extremes in their respective habitats. The *Neophasia* habitats in Arizona are only 200 km apart, yet they are drastically different with respect to winter temperatures. In the geographic range sampled, *N. menapia* experienced a wider range of thermal conditions than *N. terlooii* and significantly lower winter temperatures. Mean minimum temperatures were 4.7 °C lower and median minimum temperatures were 4.4 °C lower in *N. menapia habitat* compared to in *N. terlooii* habitat, and the all-time low was 13.9 °C lower in *N. menapia* habitat. The observed differences in SCP and LLT_{50} (respectively 7.2 and 10 °C lower for *N. menapia* compared to *N. terlooii*) are consistent with differences in minimum environmental temperature. Based on historical air temperature data from 1911 to 2013, *N. terlooii* would be ~36 times more likely to freeze that *N. menapia* in the habitat of *N. menapia*, while neither species would be likely to freeze in the habitat of *N. terlooii*. In light of the clear interspecific patterns of latitudinal adaptation, the intraspecific site effect was not significant and we therefore do not have supporting evidence of local adaptation across populations with respect to SCPs.

Our data also indicate that the thermal environments of *Neophasia* spp. are significantly impacted by microclimatic differences within and across trees. Tree microclimates were strongly correlated with air temperature but were several degrees warmer, and there was considerable variation within and among trees due to aspect and shading from surrounding vegetation. The presence of tree microclimates highlights the importance of incorporating microclimate data into

climate envelope models to accurately capture the range of thermal conditions an organism experiences (Potter et al., 2013).

Although the close correspondence of cold hardiness and minimum habitat temperature supports the hypothesis that low temperatures are setting a range limit for the southern species and maintaining a range disjunction, other factors such as plastic responses or differences in life history strategy may contribute to the differences observed between species. At their respective times of collection, *N. menapia* experiences warmer nights than *N. menapia*, and the pre-assay exposure conditions for mothers and eggs were designed to simulate this difference, thereby maximizing ecological relevance. Differences in maternal exposure could impact their offspring's thermal tolerance through maternal or epigenetic effects, but we expect this effect to underestimate differences in cold hardiness between species, because the more cold-hardy species (*N. menapia*) was initially acclimated to warmer temperatures. After initial conditions, eggs used in assays were acclimated at 5 °C for at 1–2 mo before any assays were conducted, so we expect the effects of maternal or developmental plasticity to be relatively minor compared to genetically based differences in cold hardiness. Life history strategies differ between species, with the northern species having an obligate diapause and a single generation per year compared to the bivoltine southern species. A deeper diapause in the northern species could account for differences in cold hardiness. Nonetheless, these species are closely matched in many life history traits, with similar host plant use, larval feeding behavior, egg size, and egg microhabitat, probably resulting from relatively recent divergence (Braby et al., 2007). Although we have insufficient evidence to conclude that the differences in cold hardiness maintain northern range limits in the southern species, we believe that the pattern is suggestive and the hypothesis merits further consideration, perhaps with additional Lepidoptera species with similar life histories.

5. Conclusions

We have demonstrated that overwintering eggs from butterflies in the genus *Neophasia* are freeze-intolerant and can survive temperatures down to their SCPs, with cold hardiness correlated with the extreme low temperatures in each species' respective habitat in Arizona. Temperature therefore may play an important role in setting the range limits for *Neophasia* at their near-contact zone in Arizona. However, given the low chances of either species freezing in Arizona, there may be additional mechanisms maintaining allopatry within *Neophasia*, such as competition, pathogens, and predation. Given the increasingly fragmented nature of western North American forest ecosystems, future integrative ecological studies on terrestrial "island" biota are needed to inform conservation and management initiatives, especially when considering rapid climate change.

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Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jinsphys.2018.03.005.

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